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Optimization of process conditions for infected animal tissues by alkaline hydrolysis technology

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Abstract

Alkaline hydrolysis represents a relatively new animal carcass disposal technology. It uses alkali, high temperature and high pressure to catalyze the hydrolysis of biological materials into a sterile aqueous solution and bone residues. The purpose of this work is to provide a complete study of the influence of operational parameters on alkaline hydrolysis process and to find an optimal combination of factors that maximize the disposal efficiency. In order to determine the optimal combination of the four factors *viz.* treatment time, temperature, the concentration of alkali solution and the ratio of alkali and tissue, single-factor and orthogonal experiments were used. The orthogonal experimental design included four variables (three levels) in L_9 orthogonal array. Results showed that four factors all significantly ($P < 0.01$) increased the efficiency of alkaline hydrolysis. The order of significance decreased from temperature, alkali concentration, the ratio of alkali and tissue to treatment time. Optimum process conditions were found to be as follows: treatment time, 180min; temperature, 150 ℃; the concentration of alkali solution, 5% and the ratio of alkali and tissue, 3 mL/g. The optimum conditions was validated and proven to be reliable for achieving the complete hydrolysis of different animal tissues and the inactivation of *Bacillus stearothermophilus*. This study provides a biosafety disposal method of infected animal tissues and be useful to establish effective process conditions of alkaline hydrolysis.

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Keywords: Alkaline hydrolysis; Infected animal tissue; Orthogonal design

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1. Introduction

According to statistics, the total weight of animal carcasses caused by diseases is more than two million tons in China[1]. A large number of infected animal carcasses will bring several potential hazards such as disease transmission and threat to food safety[2]. In the biological safety laboratory, the carcasses of experimental animals, which may contain virulent pathogens and toxicants, also exist huge hazards[3]. If mishandled, the pathogens will spread and threaten human health. However, traditional methods such as landfill and incineration cannot achieve the ideal biosafety disposal of infected animal carcasses.

As a new carcass disposal method, alkaline hydrolysis is a process by which heat and pressure dissolve and sterilize animal carcasses in a strong solution of sodium hydroxide (NaOH) or potassium hydroxide (KOH)[4]. The products of alkaline hydrolysis are a sterile solution, fragile bone residues and little odor[5,6]. Several studies have evaluated that alkaline hydrolysis can completely destroyed all representative classes of potentially infectious agents including prions[7-12]. Alkaline hydrolyzates are rich in nutrients and can be reused as biofertilizers[13-15] and compost additives[16,17].

As for process conditions of alkaline hydrolysis, previous studies merely used fixed parameter values[18]. A discussion involving the influence of key parameters such as treatment time, temperature and alkali concentration is lacking. Mastering the influence of parameters on hydrolysis efficiency is beneficial to the optimization of process conditions and the popularization of this technology. Therefore, the objectives of this study were to indicate the influence of operational parameters on alkaline hydrolysis process and to find an optimal combination of factors that maximize the disposal efficiency.

2. Materials and methods

2.1. Tissue digester

The experiments of alkaline hydrolysis were conducted in a tissue digester (CJKX-2, Xinyuan, China) the volume of which was 2L. The tissue digester included a sealed stainless vessel in which infected animal tissues were loaded and disposed. An electric heating jacket was installed outside the vessel. A cooling coil inside the vessel and a pump for cooling water circulation were used together to decrease the temperature in the vessel which was monitored by a sensor. A control unit could set treatment time and temperature, then carry out the process of alkaline hydrolysis. The structure of the tissue digester is shown in Fig. 1.

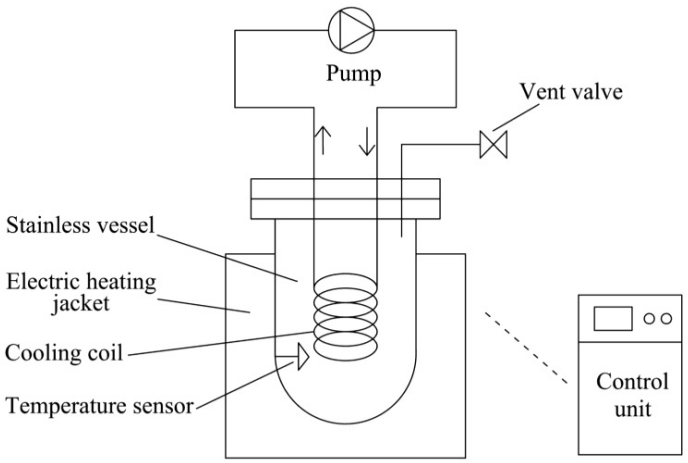


Fig. 1. The structure of the tissue digester

2.2. Alkaline hydrolysis process

For each alkaline hydrolysis process, 200g chicken (chicken breast, Dacheng, China) was weighed and loaded in the tissue digester, then the NaOH solution with designed concentration and volume was added into the vessel. After sealed, the vessel was heated by the electric heating jacket. When the desirable temperature was reached, treatment time began to count. At the predetermined time point, heating was stopped and the hydrolysis products were cooled by cooling coil. When the temperature was below 40°C, the products were taken from the vessel for measurement and analysis. In addition, the volume of NaOH solution added in the vessel was decided by the ratio of alkali and tissue which was calculated according to the Eq.(1):

$$R = V / W_1 \tag{1}$$

where R is the ratio of alkali and tissue; V is the volume of NaOH solution; W_1 is the total weight of animal tissues. Each experiment was performed in triplicates.

After alkaline hydrolysis, the hydrolysis products were divided into a liquid solution and residual tissues by filter paper. Tissue hydrolysis rate, pH value and biochemical oxygen demand (BOD) value of hydrolysis products were measured to reflect the effect of alkaline hydrolysis. Tissue hydrolysis rate was the main observing index which indicated the efficiency of alkaline hydrolysis. The following equation was used to count this index:

$$K = (W_1 - W_2) / W_1 \times 100\% \tag{2}$$

where K is tissue hydrolysis rate; W_1 is the total weight of animal tissues; W_2 is the weight of residual tissues after the disposal. The weights of initial tissues and residual tissues were measured by an electronic balance (TP-1102, Denver, USA). Besides, the acid-base property and organic materials pollution of the liquid product were shown by pH value and BOD value, respectively. The pH value was measured by a pH meter (310P, Orion, USA) and the BOD value was acquired by a BOD rapid tester (220A, Sipo Environment, China).

2.3. Experimental design and data analysis

A series of single-factor experiments were used to obtain the influence of four factors on tissue hydrolysis rate, pH value and BOD value of liquid products. Each experiment was conducted as the following conditions: (1) treatment time (60 min, 120 min, 180 min, 240 min, 300 min) by using temperature, 130 °C; the concentration of alkali solution, 1%; the ratio of alkali and tissue, 3 mL/g; (2) temperature (110 °C, 120 °C, 130 °C, 140 °C, 150 °C) by using treatment time, 120 min; the concentration of alkali solution, 1%; the ratio of alkali and tissue, 3 mL/g; (3) the concentration of alkali solution (0% (water), 1%, 3%, 5%) by using treatment time, 120 min; temperature, 130 °C; the ratio of alkali and tissue, 3 mL/g; (4) the ratio of alkali and tissue (1 mL/g, 2 mL/g, 3 mL/g, 4 mL/g) by using treatment time, 120 min; temperature, 130 °C; the concentration of alkali solution, 1%.

On the basis of single-factor experiments, a four-factor, three-level orthogonal array design (OAD)[19], L_9 (3^4) was employed for investigating the optimization of the following factors on the alkaline hydrolysis: treatment time (A), temperature (B), the concentration of alkali solution (C), and the ratio of alkali and tissue (D). Nine experiments were performed in order to estimate the best conditions for alkaline hydrolysis. Factors and levels tested are reported in Table 1.

Table 1. Variables and experimental design levels of the OAD.

Independent variables	Coded symbols	Levels		
		1	2	3
Treatment time (min)	A	60	120	180
Temperature (°C)	B	110	130	150
The concentration of alkali solution (%)	C	1	3	5
The ratio of alkali and tissue (mL/g)	D	2	3	4

The SAS Statistics software (version 9.1.3, SAS Institute Inc., USA) was used for the analysis of variance (ANOVA) of the obtained experimental data. The *p*-values of less than 0.05 were considered to be statistically significant.

2.4. Validation of optimized conditions

The efficiency of alkaline hydrolysis using optimized conditions was checked by a validation experiment. Different types of animal tissues, such as chicken breast, drumstick, pork rib and cowhide with hair, were hydrolyzed under the optimized condition. The products were observed to estimate the effect of the validation experiment.

Commercial *Bacillus stearothermophilus* (ATCC 7953) stainless coupons (1×10^6 CFU per coupon, SGM Biotech Inc., USA) were used to evaluate the sterilization ability of the optimized condition. For each sterilization cycle, four coupons were prepared. Three of the coupons were respectively put into different sterile sealed bottles which could resist the temperature of 150 °C (100ml, Dingguo, China). Another coupon was retained as a positive control outside the tissue digester. The sealed bottles were placed inside the digester and disposed in alkaline hydrolysis process. After the process, all the four coupons were aseptically deposited into universal tubes containing 10 ml of nutrient broth culture medium using sterile disposable forceps in a biological safety cabinet. Then the tubes were placed in an incubator for 7 days at 37 °C for the qualitative studies.

3. Results and discussion

3.1. Effect of treatment time on alkaline hydrolysis

The results of the single-factor experiment in which treatment time is the variable factor is shown in Fig. 2. Tissue hydrolysis rate slowly increased as treatment time increased from 60 to 300 min at a constant temperature of 130 °C, the alkali concentration of 1%, and the ratio of alkali and tissue of 3mL/g, as shown in Fig. 2(a). The highest hydrolysis rate of 84.7% was observed at 300min. When treatment time increased, the pH value decreased in Fig. 2(b) and the BOD value increased in Fig. 2(c).

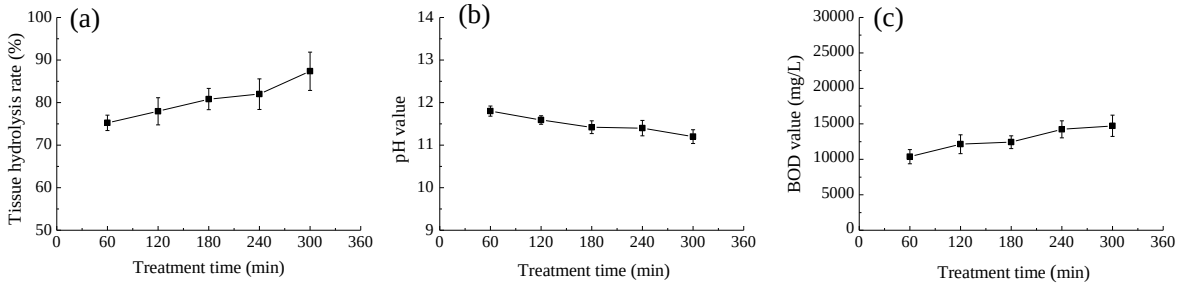
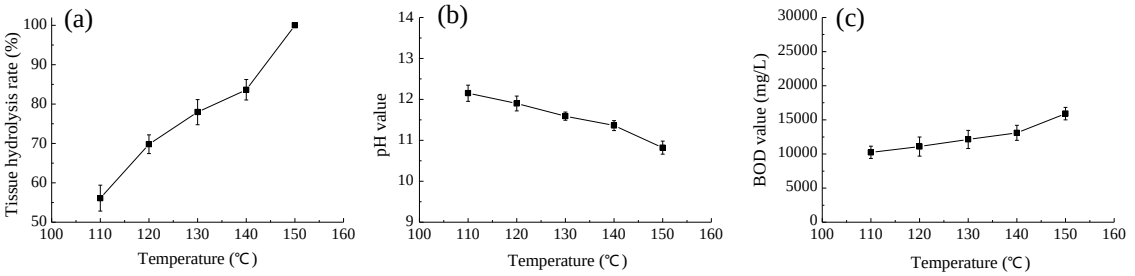


Fig. 2. (a) effect of treatment time on tissue hydrolysis rate; (b) effect of treatment time on pH value; (c) effect of treatment time on BOD value

Treatment time is a common factor that can influence the degree of hydrolysis. The increase of tissue hydrolysis rate and BOD value indicated that animal tissues were broken into soluble biological materials such as small peptides, amino acids and sugars in process. The amount of NaOH in alkali solution was consumed because of the hydrolysis reaction, pH value thus decreased from 11.8 to 11.2. However, the change of the three observing indexes was mild, tissue hydrolysis rate was also less than 100%. This phenomenon suggests that treatment time has small influence on alkaline hydrolysis.

3.2. Effect of temperature on alkaline hydrolysis

The results are shown in Fig. 3(a) that an increase in the temperature from 110 °C to 150 °C resulted in a rapid increase in the rate of tissue hydrolysis, and the rate reached a maximum value of 100% at a temperature of 150 °C. This finding suggested a positive correlation between the degree of alkaline hydrolysis and the temperature. In Fig. 3(b) and 3(c), the change of pH value and BOD value caused by different temperature was similar to the effect of



treatment time. With the increase of temperature, the pH value decreased and the BOD value increased.

Fig. 3. (a) effect of temperature on tissue hydrolysis rate; (b) effect of temperature on pH value; (c) effect of temperature on BOD value

Temperature is an important parameter which decides the rate of alkaline hydrolysis. According to the van't Hoff law[20], the rate of chemical reaction mostly increases with the rise of reaction temperature. The increase of temperature is able to accelerate the hydrolysis speed of animal tissues. Tissue hydrolysis rate is an index to estimate the degree that solid tissues resolve into soluble materials, and BOD value is a parameter to measure the number of small organics which are degraded form proteins, carbohydrate and nucleic acid in the hydrolysis product[4]. Compared to the increase speed of tissue hydrolysis rate, the BOD value grew slowly. This phenomenon indicates that temperature is conducive to the rapid dissolution of animal tissues, but plays a limited role to the degradation of proteins, carbohydrate and nucleic acid.

3.3. Effect of the concentration of alkali solution on alkaline hydrolysis

The effect of the concentration of alkali solution on alkaline hydrolysis is shown in Fig. 4. Tissue hydrolysis rate, pH value and BOD value all significantly increased as the concentration increased from 0% to 5% (0% is equal to distilled water). Fig. 4(a) shows that animal tissues were completely hydrolyzed to liquid at the concentration of 3%. High concentration of alkali solution resulted in high pH value in Fig. 4(b). The maximum BOD value of 26000 mg/L was achieved at the concentration of 5%.

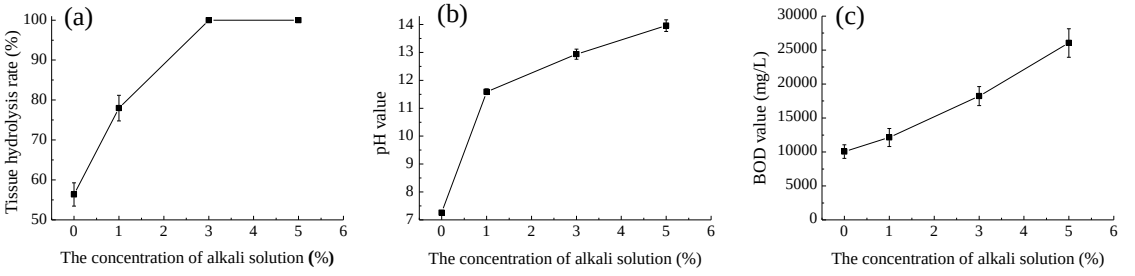


Fig. 4. (a) effect of the concentration of alkali solution on tissue hydrolysis rate; (b) effect of the concentration of alkali solution on pH value; (c) effect of the concentration of alkali solution on BOD value

The concentration of alkali solution represents the amount of NaOH used in the hydrolysis process. NaOH has a catalytic effect on the hydrolysis reaction of animal tissues. Both tissue hydrolysis rate and BOD value increased quickly with the increase of the concentration, demonstrating that the efficiency of tissue dissolution and degradation was remarkably improved by NaOH. However, excessive concentration of alkali solution would bring a lot of problems such as strong corrosion and disposal of the effluent with high pH value. Therefore, the optimization of this factor is needed.

3.4. Effect of the ratio of alkali and tissue on alkaline hydrolysis

The ratio of alkali and tissue is calculated by the volume of NaOH solution and the weight of animal tissues. As the weight of animal tissues in this study is constant, the ratio of alkali and tissue is in direct proportion to the the volume of NaOH solution added in hydrolysis process. Fig. 5(a) shows that tissue hydrolysis rate increased with an increase in the ratio of alkali and tissue. The curve of tissue hydrolysis rate increased to a greater extent at lower ratio of alkali and tissue than at higher ratio. When the volume of NaOH solution increased, the pH value increased from 10.5 to 11.9 in Fig. 5(b) and the BOD value decreased from 15720 to 9850 mg/L in Fig. 5(c). Therefore, increasing the ratio of alkali and tissue is useful to improve the efficiency of alkaline hydrolysis, but it requires sufficient volume of the tissue digester as well.

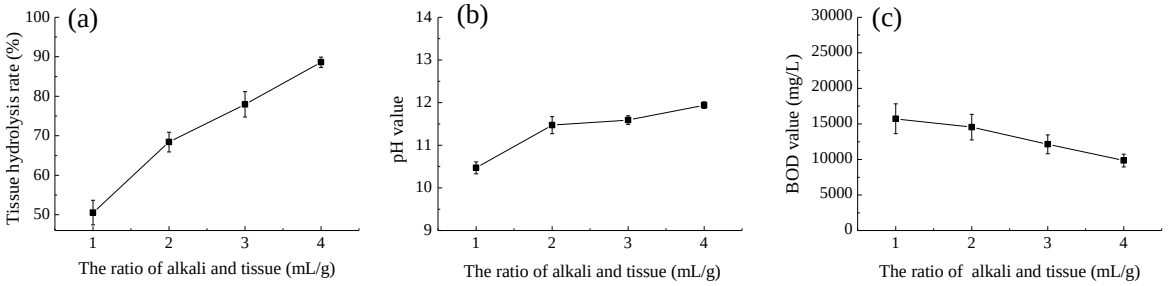


Fig. 5. (a) effect of the ratio of alkali and tissue on tissue hydrolysis rate; (b) effect of the ratio of alkali and tissue on pH value; (c) effect of the ratio of alkali and tissue on BOD value

3.5. Optimization of process conditions

The alkaline hydrolysis of animal tissues is a complex process which is affected by several factors, the optimization of the experimental conditions is a critical step in improving the alkaline hydrolysis method. Based on the single-factor experiments in present study, four different process variables *viz.* treatment time (A), temperature (B), the concentration of alkali solution (C), and the ratio of alkali and tissue (D) are considered as the most important factors of alkaline hydrolysis. A four-factor, three-level OAD was used to investigate the optimization of the four factors. Tissue hydrolysis rate, pH value and BOD value of liquid products are the indexes to judge the effect of alkaline hydrolysis. The experimental results are listed in Table 2.

Table 2. Experimental results of the orthogonal test.

Run no.	Factors				Tissue hydrolysis rate	pH value	BOD value
	A (min)	B (°C)	C (%)	D (mL/g)	(%)		(mg/L)
1	60	110	1	2	42.7 ± 3.4[a]	10.6 ± 0.3	8983 ± 765
2	60	130	3	3	98.4 ± 1.2	12.3 ± 0.1	13416 ± 709
3	60	150	5	4	100.0 ± 0	13.2 ± 0.4	10333 ± 1484
4	120	110	3	4	86.1 ± 2.0	12.8 ± 0.2	10133 ± 1341
5	120	130	5	2	100.0 ± 0	12.1 ± 0.2	20633 ± 2150
6	120	150	1	3	100.0 ± 0	10.8 ± 0.2	15040 ± 1300
7	180	110	5	3	100.0 ± 0	12.4 ± 0.3	14617 ± 1231
8	180	130	1	4	88.8 ± 2.3	10.8 ± 0.2	7450 ± 860
9	180	150	3	2	100.0 ± 0	10.9 ± 0.3	20833 ± 2980

[a] Index values are averages of three experiments, shown as mean ± standard error.

The significance of each coefficient was determined using p value. When a process variable has a p value smaller than 0.05, it influences the process in a significant way for a confidence level of 95%. In general, the effects lower than 0.05 are significant. The SAS Statistics software was used to the ANOVA of the experimental data. Results shows that treatment time, temperature, the concentration of alkali solution and the ratio of alkali and tissue contribute as significant factors for tissue hydrolysis rate, pH value and BOD value with $p < 0.05$.

It is noticed that each process condition impart different influence on the effect of alkaline hydrolysis. If the analysis is only made based on the data listed in Table 2, it was difficult to decide the best process conditions. Therefore, further range analysis was subsequently performed and listed in Table 3.

Table 3. Range analysis of the orthogonal test results.

Term	Factors											
	A			B			C			D		
	THR[d]	pH	BOD	THR	pH	BOD	THR	pH	BOD	THR	pH	BOD
M_1[a]	241.1	36.1	32732	228.8	35.8	33733	231.5	32.2	31473	242.7	33.6	50449
M_2	286.1	35.7	45806	287.2	35.2	41499	284.5	36.0	44382	298.4	35.5	43073
M_3	288.8	34.1	42900	300.0	34.9	46206	300.0	37.7	45583	274.9	36.8	27916
m_1[b]	80.4	12.03	10911	76.3	11.93	11244	77.2	10.73	10491	80.9	11.20	16816
m_2	95.4	11.90	15269	95.7	11.73	13833	94.8	12.00	14794	99.5	11.83	14358
m_3	96.3	11.37	14300	100.0	11.63	15402	100.0	12.57	15194	88.8	12.27	9305
R[c]	15.9	0.66	4358	23.7	0.30	4158	22.8	1.84	4703	18.6	1.07	7511
Optimal level	A_3			B_3			C_3			D_2		

[a] M: Sum of index for the factors at each level.
[b] m: The mean values of index for the factors at each level.
[c] $R = m$, max – m, min
[d] THR: Tissue hydrolysis rate

In Table 3, the R values indicate the effect order of the four factors, greater R values mean more obvious influence on the index. According to the R values, the effect order of tissue hydrolysis rate is shown as follows: B > C > D > A. It suggests that temperature is the most significant factor, while treatment time is the insignificant one compared with the others, which is in accordance with the results of the single-factor experiments. As for pH value, the order of impact strength is C > D > A > B. The increase of factor C and factor D brings more NaOH into the process and results in a higher pH value. Besides, the effect order of BOD value is D > C > B > A.

There is a similar result compared to the single-factor experiments by a synthesis analysis of the data in Table 3. The concentration of alkali solution has a significant influence on all the indexes, indicating an important effect on

alkaline hydrolysis. The increase of temperature can greatly accelerate the dissolution of animal tissues, but has a limited impact on the further degradation of liquid products. The small *R* values of treatment time confirm minor influence on alkaline hydrolysis.

Since the objective of alkaline hydrolysis is to convert solid animal tissues into liquid products and accomplish biosafety disposal, tissue hydrolysis rate is regarded as the main index to optimize process conditions. The level of four factors which obtained the highest main value of tissue hydrolysis rate was chosen as optimized process condition. The combination of factors found after the calculation as optimal was $A_3 – B_3 – C_3 – D_2$ *viz.* treatment time, 180 min; temperature, 150 °C; the concentration of alkali solution, 5%; the ratio of alkali and tissue, 3 mL/g.

In addition, the highest value of treatment time, temperature and the concentration of alkali solution was appeared in the optimized conditions. Further increase of these factors might acquire a more effective alkaline hydrolysis process. However, increasing temperature equals to a higher operating pressure which will increase manufacture difficulty and cost of the tissue digester. The increase of treatment time has a limited effect on the process. Higher concentration of alkali solution remains the problem that liquid products with high pH value need further disposal. Therefore, considering the experimental results and simple application, the optimized process conditions were determined as above.

3.6. Validation of optimized conditions

In present study, a confirmatory experiment was conducted to probe the reliability of the results obtained. Four types of animal tissues *viz.* chicken breast, drumstick, pork rib and cowhide with hair were disposed under the optimized conditions. The weight of each type of animal tissues was 200g. The results of the validation experiment is shown in Table 4. Different types of animal tissues were all completely hydrolyzed in the optimized conditions. The products were dark liquid and bits of fragile bone residues.

Table 4. Results of validation experiments by different types of animal tissues.

Type of animal tissues	Liquid product	Solid product
Chicken breast	Dark yellow liquid	None
Drumstick	Dark yellow liquid	Fragile bone residues
Pork rib	Dark yellow liquid	Fragile bone residues
Cowhide with hair	Coffee-colored liquid	Grey dregs

In order to verify the sterilization ability of optimized conditions, an experiment to inactivate the biological indicator of *B. stearothermophilus* was conducted in optimized conditions. In Table 5, the results showed that the liquid culture medium in tubes containing coupons in the experiment exhibited no growth after seven days of incubation. All the positive controls turned cloudy and showed growth. The optimized conditions could achieve a complete inactivation of *B. stearothermophilus.*

Table 5. Results of sterilization experiments.

Observation point	Experiment group			Control group
	No.1	No.2	No.3	
The first day	(−)[a]	(−)	(−)	(+)[b]
The third day	(−)	(−)	(−)	(+)
The seventh day	(−)	(−)	(−)	(+)

[a] (−): the coupon showed no growth.
[b] (+): the coupon showed growth.

4. Conclusions

In this study, the influences of treatment time, temperature, the concentration of alkali solution and the ratio of alkali and tissue on alkaline hydrolysis were evaluated by single-factor experiments in order to develop an optimized process. L_9 (3^4) orthogonal array design was successfully applied for optimization of the efficiency of alkaline hydrolysis. The optimized conditions were listed as follows: treatment time, 180 min; temperature, 150 °C; the concentration of alkali solution, 5%; the ratio of alkali and tissue, 3 mL/g. We also concluded that the optimized conditions could accomplish complete hydrolysis of different types of animal tissues and significant inactivation of *B. stearothermophilus*. Consequently, the optimized conditions can meet the requirements of the biosafety disposal of infected animal tissues and be reliable for practical application. Alkaline hydrolysis technology provides a new way to the biosafety disposal of infected animal carcasses and the safe treatment of hazard waste in laboratory. The findings in the paper can be used as a reference for the application of alkaline hydrolysis and may be helpful for the further popularization of this technology.

Acknowledgements

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